Microbial Volatilization: Bioremediation of Soils Contaminated with Arsenic

A Senior Honors Thesis

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By

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INTRODUCTION

Arsenic (As) is found naturally in soils throughout the world. Although the metalloid has a concentration of only .0001% in the Earth's crust, it has often been the focal point of many scientific studies due to its classification as both a carcinogen and a poison (Oremland, 2003).

As is most often found in sulfides such as realgar and orpiment (Matera, 2002). It exists in a number of oxidation states, with the most environmentally and biologically relevant species being arsenate, As(V), and arsenite, As(III). Arsenate is toxic, but adsorbs strongly to many common minerals, limiting its mobility. Arsenite on the other hand is relatively mobile in the environment, and is about one-hundred times more toxic than arsenate (Weeger, 1999).

Volatilization Biochemistry and Pathways

Volatilization is the process in which certain species of fungi and bacteria methylate inorganic As species to form methylarsenicals (Rodriguez, 1998). After their formation, methylarsenicals are released from the microbe as a gaseous product (Fig. 1). Volatilization is a natural process that occurs globally, and species capable of As volatilization have been isolated from many diverse environments, including soils, rivers, hot-springs, and even the human intestine (Turpeinen, 1999, Kaise, 1997, and Jackson, 2001). The substrates acted upon by these microorganisms, as well as the volatile products released, typically vary from organism to organism.

Many diverse microorganisms are capable of As volatilization. Some anaerobic methanogenic bacteria are capable of reducing inorganic arsenate to arsenite. They can then convert either arsenite or methanearsonic acid to gaseous dimethylarsine through

volatilization pathways (McBride, 1978). *Escherichia coli*, which is common in the intestines of humans and other animals, can form volatile trimethylarsine (Frankenberger, 2002).

More importantly, from a bioremediation perspective, several species of soil dwelling microorganisms have shown As volatizing potential (Frankenberger, 2002). This includes species of *Penicillium* and *Aspergillus,* which can volatilize both organic and inorganic As compounds, and *Pseudomonas*, which are capable of volatilizing inorganic As. Thus, the natural presence of soil dwelling As volatizing organisms offers the possibility to stimulate bioremediation of As contaminated soils.

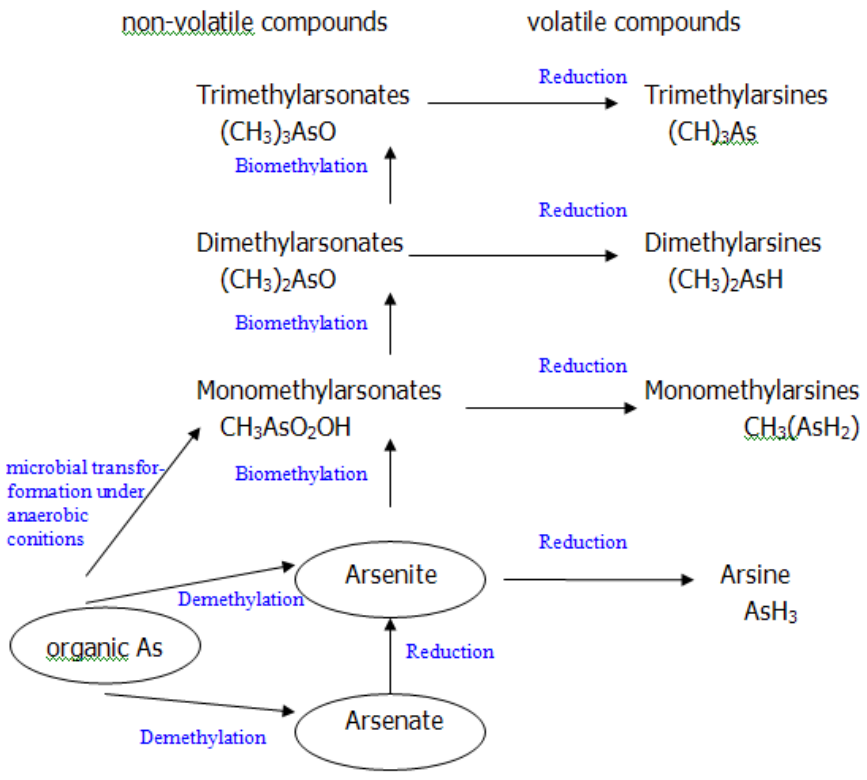


Figure 1: Arsenic volatilization pathways (all processes are reversible)
modified from Sadiq (1997) and Frankenberger and Arshad (2002)

Bioremediation by Volatilization Mechanism

Manipulating Biological Agents One of the first documented studies of As volatilization was carried out by the Italian physician Bartolomeo Gosio (Bentley, 2002). In 1891, Gosio found that a series of As poisonings occurring in Italy were caused by the volatilization of As. The source of the As poisonings were the dyes Scheele's green and Schweinfurth's green, which were both commonly used in wallpaper at the time. Gosio found that when he grew the fungus *Penicillium brevicaule* on the wallpaper, the colonies produced a foul smelling, As containing gas. Gosio also found that when rats were placed in a chamber that contained the colorless, garlic-odored "gosio gas", they quickly died (Bentley, 2002).

Although Gosio had proven that As volatilization was carried out by microorganisms, there was still much debate over the mechanism by which volatilization occurred. In the 1940s, Challenger proposed a mechanism for volatilization by the fungus *P. brevicaulis* (Challenger, 1945). The method, now known as the Challenger Mechanism, proposed that arsenate undergoes three sequential reductions, and three methylations by the enzyme SAM, to form trimethylarsine (Fig. 2).

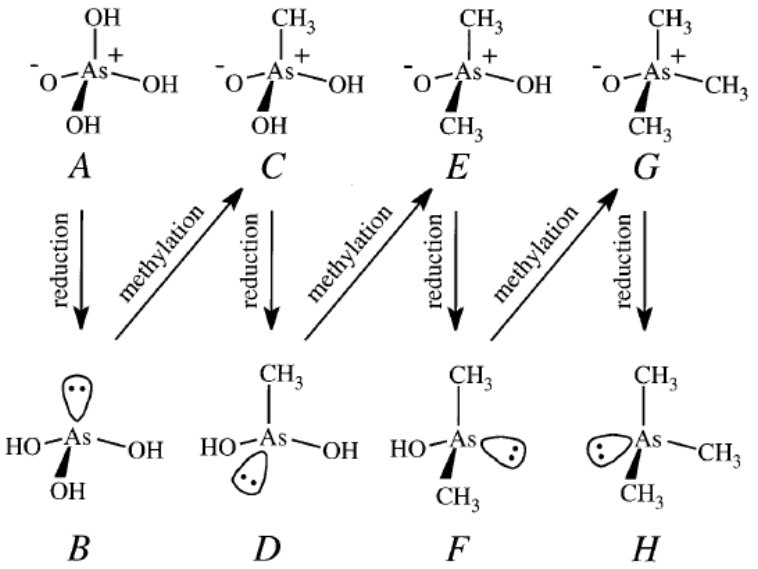


Fig. 2 Challenger Mechanism of volatilization in the fungus *P. brevicaulis*. Taken from Bentley (2002).

Scientific literature shows that the rate of As volatilization in soil communities varies widely. Some recent experiments have reported the rate of As volatilization to be only .0005 % of total As content over a twenty week period (Rodriquez, 1999), whereas others have reported volatilization rates of over 10 % (Hassler, 1984). The large discrepancy in volatilization rates is most likely attributable to varying soil conditions and differing microbial communities.

Chemical/physico Factors of Volatilization Volatilization rates can be affected by a variety of chemical/physical factors. Soil pH, moisture, and temperature, as well as the addition of organic matter, heavy metals, and phosphorus to soils, all have effects on the rate of volatilization observed. Some of these conditions affect volatilization rates by simply promoting or inhibiting microbial growth.

Others have a more complex effect on volatilization. For example, phosphorous is an important nutrient necessary for the growth of microbes, but when added to colonies of volatilizing bacteria, phosphorous leads to the inhibition of trimethylarsine formation

(Cox, 1972). This phenomenon is most likely explained by the fact that As and phosphorous are chemical analogs. Because of the similarity between the two elements it is probable that As species enter cells through the phosphate transport system, leading to competition between the uptake of phosphorus and As (Kertulis, 2004).

Volatilization can occur under both aerobic and anaerobic conditions. Soil dwelling organisms, such as *Penicillium,* tend to exhibit higher levels of volatilization under anaerobic conditions (Frankenberger, 2002). This is due mainly to the fact that As(III), the most common substrate of volatilization, is more plentiful under reduced conditions.

Rates of volatilization are also affected by the form of As substrate. Each volatilizing organism has its own list of substrates it is capable of transforming. In general, volatilization rates are much higher for organic arsenicals as opposed to inorganic ones (Frankenberger, 2002).

Temperature has a large effect on most biological processes, including volatilization. Frankenberger found that the optimal temperature for volatilization in *Penicillium* was 20ºC (Frankenberger, 2002). It follows that the optimal temperature for volatilization will correspond to the temperature at which the given organism has the highest growth rate, as enzymatic rates are highest under these conditions.

Environmental pH is also an important factor for volatilization rates. Most studies have shown the process to occur most efficiently in acidic conditions, around pH of 5.0 (Baker, 1983). This phenomenon probably has less to do with biological processes and is more aptly attributable to mobilization of As compounds. As species are generally more mobile under acidic conditions, making them more readily available to

volatilizing organisms (Cox, 1972).

The presence of metals in soil can have a positive or negative affect on volatilization. For example, chromium concentrations of ~100 μM have been shown to strongly inhibit volatilization, whereas similar iron concentrations have been shown to promote it (Frankenberger, 2002). Many other metals have been shown to have similar consequences of promoting or inhibiting volatilization rates.

In order to develop As bioremediation technologies based on natural attenuation, it is critical that the environmental conditions that control microbial As volatilization be understood.

Organic Amendments Organic amendments have been shown to affect the rates of volatilization in soils, presumably by stimulating microorganisms. The use of organic material to promote volatilization is desirable because it could recycle organic wastes, improve soil quality, and is compatible with a society that is demanding natural bioremediation methods. Studies have shown that soils with ~11 % organic matter have the highest rates of As volatilization (Akins, 1976).

Additions of glucose directly to soil showed increased As volatilization under aerobic but not anaerobic conditions (Frankenberger, 2002). Additions of carbohydrates always lowered volatilization rates, while adding amino acids had either positive or negative effects depending on the specific amino acid used (Huysmans, 1990). Additions of fresh manure to soil may also increase the bioavailability of As species that appear to be linked to higher rates of volatilization (Walker, 2004).

Soil moisture level has a distinct effect on volatilization rates as well. In studies by Gao, soil samples exhibited optimum moisture levels around 250g H_2O kg^{-1} soil (Gao,

1997). Increases in soil moisture level above this value led to a decrease in volatilization rates. Very dry soils also showed depressed volatilization levels.

PROJECT RATIONALE

There is widespread interest in remediation techniques for As contaminated sites. Biologically toxic levels of As can accumulate in water or soil through natural processes, as well as being the result of human practices, such as the smelting of metals and the use of As containing pesticides (Cullen, 1989 and Rodriguez, 2003).

Two of the most common routes of As poisoning are through the drinking of contaminated ground water and the accidental ingestion of contaminated soil (Buschmann, 2008 and Baptist, 2008). Contamination of ground water usually occurs when As is leached into water systems that lead to a common underground pool. As accumulates in these pools which are often tapped by community wells. Because these wells are used by entire communities, the risk of mass poisoning is very serious. This scenario is occurring right now in Bangladesh and other areas of the world (Van Geen, 2005).

A major issue concerning As contamination now is the residual effects of wood that has been treated with As as a preservative. Although As preservatives are no longer used to treat wood, old structures such as fences, house decks, and children's play sets continue to contaminate soils (Cullen, 1984 and Baptist, 2008). Accidental ingestion of As contaminated soils is particularly problematic among young children (Rodriguez, 2003). Consequently, there is growing concern regarding As in the environment because of the previous large scale use of As treated wood.

Given the toxicity of As to humans and other life forms, it is imperative that

inexpensive, effective, and safe methods of As remediation be developed. Although remediation techniques such as the mechanical removal of contaminated soil and the planting and harvesting of As accumulating plants are currently being used, these methods are often too costly, ineffective and invasive (Lambert, 2003 and Tripp, 1996). A remediation method that may prove to be an effective, safe and economical way of removing As is microbial volatilization.

In order for volatilization to function as an efficient bioremediation technique, efforts must be made to maximize the rate at which it occurs in the field. By treating As polluted soils with inexpensive amendments, volatilization may prove to be an effective bioremediation method. However, there are few studies on practical, field-based methods for bioremediation of As contaminated soils. Therefore the objective of this study was to determine the impact of organic and inorganic soil amendments on extractable As levels and volatilization of As in contaminated soils.

MATERIALS AND METHODS

The laboratory setup consists of twenty-four bioreactors designed to monitor gaseous emissions of As from soil. The design was a completely randomized experiment with four treatments; vermicompost, glucose, $Ca_3(PO_4)_2$, and bread crumbs, as well as two controls, one with and one without the addition of As, with four replications per treatment. Soils were spiked with 1000 ppm As in the form of As_2O_3. The vermicompost, glucose, and bread crumb treatments were added at 4000 μg C g^{-1} soil. The $Ca_3(PO_4)_2$ amendment was added at 100 μg P g^{-1} soil. For each replicate, two separate 50 mL beakers, one containing 50 mL of 0.1 M KI and another containing 30g of As spiked soil, were placed in 1L mason jars, which were immediately capped with an

airtight lid after the amendments were added. The soil used was a Muscatune Silt Loam, with a carbon content of 2.68%. All soil moisture was adjusted to 2/3 field capacity.

Bioreactors were incubated for 14 days at 20°C. At t=0 and t=14 days, water extractable As was measured. At t=14 days, volatilized forms of As were measured using ICP analysis of the KI solution. Community fatty acid methyl ester (FAME) profiles were determined at t=14 to compare microbial community structure (Shutter & Dick, 2001).

RESULTS and DISCUSSION

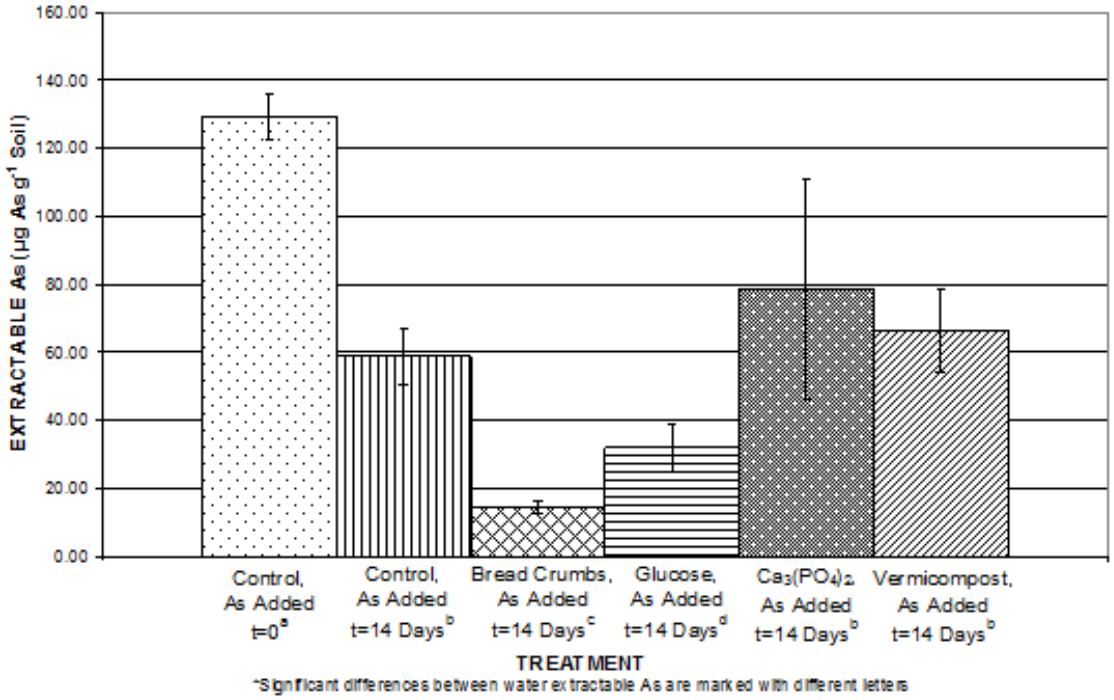
The static approach for trapping volatile As gave inclusive results (data not shown). For the most part, As levels across treatments were below the detection limit. One replication of the bread treatment had a value of less than 10µg As evolved g^{-1} soil, whereas another showed 943µg As evolved g^{-1} soil. The inclusive results would indicate it is necessary to have a gas flow through the As trapping system in order to effectively capture volatile As compounds. Alternatively, it may be that a longer incubation time is needed under the conditions of this experiment.

Water extractable As decreased in all four treatments, as well as the spiked control, over the course of 14 days (Fig. 3). Loss of water extractable As may indicate that As is leaving the soil through volatilization. Also it is very likely As is being immobilized through biological activity into soil organic matter or chemical fixation that makes it unextractable.

The bread crumb and glucose treatments showed significant reductions in water extractable As at t=14 days compared to the control, indicating that volatilization, immobilization, and fixation may have been stimulated by these treatments. The

$Ca_3(PO_4)_2$ and vermicompost treatments exhibited higher extractable As levels at t=14 days than the other amended soils, meaning volatilization, immobilization, and fixation were less effective in reducing extractable As in these two treatments

Fig. 3:
Effect of Soil Amendments on Water Extractable As



*Significant differences between water extractable As are marked with different letters

EL-FAME analysis of the microbial communities at t=14 days showed that the treatments with the lowest water extractable As levels, bread crumbs and glucose, had a lower bacterial:fungal ratio, meaning a larger percentage of their communities were composed of fungi compared to other treatments (Table 1). Soil communities treated with bread crumbs and glucose also showed depressed levels of actinomycetes, as well as other Gram+ bacteria, compared to the other treatments. Glucose treatments also exhibited lower levels of Gram– bacteria. The control, vermicompost, and $Ca_3(PO_4)_2$ treatments shared similar microbial community structure in every category. These two treatments showed bacterial:fungal ratios that were much lower than the bread crumb and

glucose treatments.

Table 1: Fatty Acid Biomarker Composition by Amendment (% of total fatty acids)

	Control (No As)	Control (As Added)	Bread Crumbs	Glucose	$Ca_3(PO_4)_2$	Vermicompost
Total Fungi	22.47 ± 1.38	20.76 ± 0.49	38.62 ± 3.73	32.50 ± 2.55	20.60 ± 0.96	22.42 ± 0.82
Total Bacteria	76.06 ± 1.68	78.39 ± 0.72	59.68 ± 3.65	66.70 ± 2.57	77.95 ± 2.93	75.34 ± 2.02
Gram +	16.05 ± 0.38	16.24 ± 0.81	8.11 ± 1.87	9.59 ± 0.88	15.73 ± 0.18	15.07 ± 1.44
Gram -	15.23 ± 0.30	15.41 ± 0.61	15.53 ± 2.84	7.97 ± 2.20	14.61 ± 0.45	15.45 ± 0.89
Actinomycetes	8.91 ± 0.17	9.07 ± 0.35	2.66 ± 0.48	4.24 ± 0.59	8.33 ± 0.41	7.36 ± 0.48
Protozoa	1.47 ± 0.85	0.85 ± 0.28	1.71 ± 0.40	0.50 ± 0.11	1.45 ± 2.04	2.23 ± 2.84
B:F Ratio[1]	3.40:1	3.78:1	1.56:1	2.07:1	3.79:1	3.36:1

1. Ratio of total bacterial biomarkers to total fungal biomarkers.

The As spiked control showed a reduction in water extractable As over the course of 14 days. It is very likely that a significant amount of the added As was immobilized or fixed in the soil matrix, making it unextractable. Also, volatilization may have been occurring at low levels, as it has been shown to occur naturally in soils that are left untreated. This rate of volatilization, however, may be too slow to be considered acceptable for bioremediation techniques.

The bread crumb treatments showed the lowest level of water extractable As, and thus the highest rate of volatilization, immobilization, or fixation. The microbial response did provide indirect evidence for this treatment to increase As volatilization because it had the highest proportion of fungi compared to the other treatments. Previous findings have shown certain fungi, most notably *Scopulariopsis bevicaulis* and *Phaeolus schweinitzii,* to volatilize As_2O_3 (Pearce, 1998). Qualitative visual observations showed the bread crumb treatment having a dense fungal mat on each of the four replicates. This increased community size may have contributed to a faster rate of As volatilization, immobilization, or fixation.

The glucose treatments showed the next highest levels of water extractable As.

Reports from the literature would suggest glucose can stimulate As volatilization, which may be the case in this experiment. Studies by Thomas and Rhue showed that the addition of glucose had no effect on volatilization under low oxic conditions, but increased volatilization under high oxic conditions (Thomas, 1997). Glucose may have stimulated As volatilization in this study by promoting microbial growth, leading to a higher concentration of volatilizing microorganisms. The glucose treatment also exhibited a low bacterial:fungal ratio, although not as low as the bread crumb treatments. Glucose was not as effective in stimulating reduction in extractable As as the bread crumb treatment was, mainly because glucose is quickly metabolized by microorganisms and is rapidly depleted as an energy source. Also, it is likely that immobilization and fixation stimulated by the glucose amendment played a significant role in reducing extractable As.

The $Ca_3(PO_4)_2$ treatments showed increased levels of water extractable As compared to the control. As mentioned above, studies have shown that high phosphorous levels may interfere with a cell's uptake of As (Cox, 1972). Because As is not able to enter the microbe, it cannot be volatilized. This competition between As and phosphorous explains the lower rate of volatilization among $Ca_3(PO_4)_2$ treatments. Also, $Ca_3(PO_4)_2$ could through chemical reactions displace As into soil solution by competing with sorption sites and other reactions. In this case PO_4 competes with As species for the same sorption sites in soils and therefore maintains higher levels of As in soil solution.

The vermicompost treatment also led to decreased levels of water extractable As. The vermicompost showed a unique microbial community structure. Many fatty acids that appeared in the vermicompost treatments did not appear at all in the others. These

unique organisms may have interfered in some way with the volatilizing organisms in the soil, perhaps by competing with them for nutrients, leading to a lower level of extractable As. The vermicompost may also have interfered with the immobilization and fixation that was promoted by the bread crumb and glucose treatments.

The results of this experiment show that bread crumbs and glucose treatments may be effective methods to increase volatilization, immobilization, and fixation during bioremediation of As in contaminated soils. $Ca_3(PO_4)_2$ and vermicompost treatments, on the other hand, should most likely be avoided as sole applications during bioremediation efforts. It may be that combination of $Ca_3(PO_4)_2$ with an organic treatment such as bread crumbs could have a synergistic effect, whereby the $Ca_3(PO_4)_2$ keeps As in soil solution and makes it more bioavailable for the populations stimulated by bread crumbs. In turn this could further increase As volatilization over bread crumb treatments by themselves.

The study also suggests that the most efficient method for improving volatilization rates is to increase the concentration and percentage of fungi in contaminated soil communities. By amending contaminated soils with inexpensive amendments that stimulate fungal growth, such as bread crumbs, volatilization may prove to be an effective and efficient means of bioremediation.

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